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***In vitro* Regeneration, Acclimatization and Antimicrobial Studies of Selected Ornamental Plants**

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Additional information is available at the end of the chapter

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1. Introduction

Tissue culture has been applied to diverse research techniques such as viral elimination, clonal propagation, gene conservation, *in vitro* fertilization, mutation, induction for genetic diversity, genetic transformation, protoplast isolation and somatic hybridization, secondary metabolite production and other related techniques. The commercial production of ornamental plants is growing worldwide. Its monetary value has significantly increased over the last two decades and there is a great potential for continued further growth in both domestic and international markets. About 156 ornamental genera are propagated through tissue culture in different commercial laboratories worldwide. About 212.5 million plants including 157 million ornamental plants amounting to 78% of the total production were reported [1]. These plants are over exploited due to their high medicinal value and hence, propagation of the plants by tissue culture may be mandatory, which offers a greater potential to deliver large quantities of disease-free, true-to-type healthy stock within a short span of time. Biotechnological interventions for *in vitro* regeneration, mass micropropagation and gene transfer methods in forest tree species have been practiced with success, especially in the last decade. Against the background of the limitations of long juvenile phases and lifespan, developments of plant regeneration protocols of ornamental species are gaining importance. Ornamental industry has applied immensely *in vitro* propagation approach for large-scale plant multiplication of elite superior varieties. During *in vitro* condition, plantlets are grown under fixed and controlled environment in sterile formulated medium which contained macronutrients, micronutrients, vitamins and plant growth regulators. After the plantlets reached optimum growth in the culture containers after a certain growth period, it can be transferred to *ex vitro* condition to allow continuous growth of the plantlets. As a result, hundreds of plant tissue culture laboratories have been set up worldwide, especially in the developing countries due to cheap labour costs.

Plant tissue culture media is normally rich in sucrose and other organic nutrients that can support organogenesis in plants but also the growth of many microorganisms (like bacteria and fungi). To overcome and prevent contamination in media preparation, sterilization should be done thoroughly. Sterilization of nutrient media can be done in an autoclave (large pressure cooker), less often by filtration and seldom by irradiation [2]. The container with the medium should be properly closed and autoclaved at 121°C, 105 kPa, for 20 minutes. It also identified that good sterilization relies on time, pressure, temperature and volume of the object to be sterilized [2]. The sterilized nutrient media should be stored in a sterile box that has previously been disinfected with 70% alcohol [2]. Some of the plant growth regulators such as gibberellic acid (GA_3), zeatin, abscisic acid (ABA), urea, certain vitamins, pantothenic acid, antibodies, colchicines, plant extracts and enzymes used in tissue culture is thermolabile. These compounds should not be autoclaved and filter-sterilization is often used if a thermolabile substance is needed in a nutrient medium.

2. Problem statement

In vitro plant tissue culture needs the formulation of a complete nutritional medium and for exploration of plant physiological processes; it needs the addition of effective plant growth regulators. These two aspects can be considered to plant tissue culture the wings to take off. With the starting of common or specific media and the selection of appropriate plant tissue culture, enable induction of cell division, callus growth, differentiation of shoots, roots and embryos. Commonly, synthetic analogues are used, mainly; 1-naphthalene acetic acid (NAA), indole butyric acid (IBA), 2,4-Dichlorophenoxyacetic acid (2,4-D) in plant tissue culture for the induction of plant cells and the regeneration of the root, bud, embryo-like body which has a high organizational structure of specialized cells. The main physiological role of cytokinins; promotion of cell division and expansion, buds induction, differentiation, promote lateral bud sprouting and inhibition of senescence. *In vitro* tissue or organ will be soon aging. Such as cytokinin treatment can slow the aging process. Another class of hormones used in tissue culture is gibberellins. The main physiological effects of gibberellin (GA) are buds induction. It is commonly used in plant tissue culture to promote the growth of the seedling stem elongation. It is also affect cell differentiation of cambium and it often works in synergy with auxins. In addition, the other hormone such as abscisic acid (ABA) is one of the plant growth substances; it can be biosynthesized from mevalonic acid. It can control water and ion uptake by roots and to promote the adventitious shoots and absorb and prevent the phenolic production. Whereas, the ethylene one of the gases plant hormone, it is moved by diffusion around the plant rather than translocation. It has stimulates the final stage of fruit development and flower fall. The main function of ethylene in plant tissue culture, it can stimulate the respiration, seed germination, peroxidase enzymes and regulates the level of auxins. The low concentration of ethylene induces the proper resistance to the developed plant.

3. Application area

The physiological role of auxins promote cell growth and cell division, induction of the injured tissue of one to several layers of cells recovering the ability to divide to form

callus; promote rooting, in the conventional cutting propagation and in organized tissue culture. Auxins can also promote plant sex differentiation, the formation of flowers and to promote the formation of seedless fruit. Auxins main use in tissue culture to induce callus and, also they are important use is in line with a certain amount of cytokinin for root differentiation, lateral bud germination and growth, as well as in certain plants induce embryogenesis. Since, many research articles have dealt at great length about the status, applications, potentials and needs in tissue culture of ornamental plants. Since, the major effects have been made to develop basic back ground technologies for consistent production and regeneration of calluses from diverse group of medicinal plants [3]. The techniques which have been so far described for propagation of ornamental plants through tissue culture have been tested on the laboratory scale and have not been validated for their suitability in commercial scale production. However, the following aspects have to be critically studied if the economic prospects associated with *in vitro* culture technology are to be realized. Generally, the application of plant cell and tissue culture technique which is commonly known as *in vitro* cloning can also be divided into several procedures, including meristematic cultures, vegetative explant cultures, callus induction, suspension cultures, direct and indirect somatic embryogenesis, synthetic seed production, *in vitro* flowering, *in vitro* mutation breeding, protoplast and also somatic hybridization process. Some of these techniques would selectively applied to selected plants overcome generation incapability of the plants. This *in vitro* technique is very useful in ensuring sustainable, optimized sources of plant-derived natural products. However, *ex situ* cultivation should be preceded by proper evaluation of the plants for their ability to produce the required bioactive constituents before commencing cultivation or introducing the technology to potential growers. The ability of plants to produce certain bioactive substances is largely influenced by physical and chemical environments in which they grow. Plants also produce certain chemicals to overcome abiotic stresses. In this aspects plant tissue culture developed callus influenced by medium, explants, plant growth regulators, color lights, temperature, photoperiod and carbon sources are helpful to produce valuable secondary metabolites compounds in many studies [3,4]. Growing a plant outside its natural environment under ideal conditions may therefore, result in being unable to produce the desired bioactive substances, hence the need for prior evaluation.

4. Research course

Ornamental plants are used especially as decorative houseplants and for landscaping. Ornamental plants are unique for their sheer beauty and variety of leaves. For example Begonias, which have a medicinal value. It is a temperate plant, which is commercially used as a flowering pot plant. It does not produced seeds. It is a winter flowering plant and was developed from cross between other species of the same family. Begonia plant normally has thick, shiny, dark green leaves. Although Begonias can be readily vegetative propagated, they are susceptible to many pathogenic bacteria, fungi, and nematodes [5]. Nowadays, the seaweed market has grown as predicted with prospects to go even further. Therefore

potential improvements introduced through the application on *in vitro* techniques are expected to be even higher.

Modern techniques of propagation through tissue culture technique have been developed to meet the demand of the horticultural industries including nursery industries. For pot plant production, the priority is to obtain early, synchronized and profuse flowering, together with a compact and homogenous plant size, rather than continuous flowering. For ornamental pot plant production to be successful, an efficient method for flower induction in small plantlets is thus required. Thus, the application of plant tissue culture technique is always required. The technology is widely applied in both research and development of improved crops [6]. Rout *et al.* reported that about 156 ornamental genera were propagated through tissue culture in different commercial laboratories worldwide [7].

5. Method used

5.1. Surface sterilization

Surface of plant parts carry a wide range of microbial contaminants. The presence of any contaminant will interfere with the growth of explant or cultures and fungal or bacterial explant contamination in plant cultures is usually detectable 1-14 days after culturing. Therefore, sterilization or disinfection of tissues is necessary in order to eradicate surface microorganism. In order to disinfect plant tissues, 5-50% (v/v) commercial bleach Clorox (Sodium hypochlorite), 70% (v/v) alcohol and a few drops of Tween 20 can be used in sterilization technique. Pierik *et al.* had suggested that sterilization plant for a few seconds in alcohol is not sufficient to kill all microorganisms and after this they are usually treated with sodium hypochlorite [2]. Diluted solution of sodium hypochlorite (0.25-2.63%) (v/v) is used as a disinfectant and tween 20 is an emulsifier which is added at the rate of 1 drop per 100 ml of solution. The development of techniques for the culture of isolated plant organs, tissues and cells have led to several exciting opportunities in the area of plant biotechnology, and allowed widespread use of cell culture for *in vitro* genetic manipulation, plant propagation and production of commercially useful products. The techniques of cultivating cells and tissues have been referred to sometimes as “aseptic culture of plants”. Therefore, the absence of contaminants is assumed to be a fundamental requisite *in vitro*. Surface sterilization of ornamental plants is difficult as they lack a thick protective surface, and therefore sodium hypochlorite and similar agents can easily damage the delicate tissues. The scope of these techniques has been extended for use in bioprocess technology for production of high value chemicals of immense commercial value in the pharmaceutical and nutraceutical sectors [8]. Finally, the instruments including forceps and scalpels were sterilized by dipping them into hot bead sterilizer at 250°C and allowed to cool. Glassware, empty test tubes, empty flasks, petri dishes, filter paper and distilled water can be sterilized using an autoclave at 121°C, 105 kPa, for 20 minutes. The bottles and glassware should not be too tightly packed and their tops should be loosen during autoclaving.

5.1.1. Explants sterilization procedure

To initiate cultures, various explants of African violet (*Saintpaulia ionantha* H. Wendl) were excised from 2-month-old intact plants which were grown in greenhouse. The flower buds (3-5 mm), leaf (10 x 10 mm), petiole (10 mm) and peduncle (10 mm) were used as explants for *in vitro* studies [9]. In some cases aseptic seedlings need to be used. Seeds of *Dianthus caryophyllus* were washed with chlorox concentration of 70%, 50%, 30%, 20% and 10% (v/v). First, the seeds were shaken in 70% chlorox with three drops of tween-20 for 15 minutes. They were then washed three times with sterile distilled water. These steps were repeated with other concentrations of chlorox. The seeds were cultured into test tubes containing MS media without hormone under aseptic condition. The work has to be done under sterile conditions. The seeds were germinated in a culture room at the temperature of 25±1 °C with 16 h light and 8 h dark. The growth was evaluated after 30 days. For tissue culture studies of *Gerbera jamesonii* Bolus ex. Hook f. various explants were obtained from 8-week-old aseptic seedlings. *Gerbera* seeds were first soaked in distilled water for 30 min with addition of 1-2 drops of Tween-20, followed by 40% (v/v) Sodium hypochlorite solution and gently agitated. The seeds were then rinsed 3 times with distilled water and then soaked in 70% (v/v) alcohol for 1 min. Finally the seeds were rinsed 3 times with sterile distilled water. Sterilized seeds were cultured in MS basal medium [10]. Surface sterilization process for seeds of cauliflower (*Brassica oleracea* var. *botrytis*) was slightly different; the seeds were soaked in distilled water with 1 or 2 drops of Tween-20 for 20 min, followed by 60% (v/v) Sodium hypochlorite solution, gently agitated for 15 min. The seeds were then rinsed 3 times in distilled water, soaked in 70% ethanol (v/v) for 30 sec and rinsed again in 3 changes of sterile distilled water prior to culturing in MS basal medium [11]. The conventional methods of propagation are problematic due to rapid occurrence of diseases. The production of large numbers of genetically homogenous plants is also very difficult. Plant cell culture technique is an alternative method for mass cloning of Begonia plants and also to overcome the problems occurring in the conventional propagation. The regeneration frequency and average number of shoots per explant varied among the cultivars. Shoot tip size also plays an important role in shoot regeneration efficiency [7]. Wang and Ma reported that shoot tip between 0.2 and 0.5 mm and shoot meristems between 0.1 and 0.2 mm diameter produced only a single shoot [12]. The techniques of stimulating axillary branching or culturing nodal sections *in vitro* are probably most commonly used in micropropagation [13]. Size of the meristem (both shoot tip and nodal explants) of *Floribunda* and miniature roses had significant effect on shoot multiplication; on an average 2.5-5.0 shoots were obtained per culture cycle, dependent on cultivars [14]. Recently, Teixeira de Silva and Fukai published a detailed review on tissue culture of chrysanthemum, which highlights organogenesis, thin cell layer, and somatic embryogenesis for plant regeneration [15].

5.1.2. Media and plant growth regulators roles

MS basal medium [16] was used for these experiments. The constituent of the media was adjusted to 1 liter after the sucrose addition and the pH was adjusted to 5.7 prior to the adding of 7.0 g agar and 0.1 g charcoal to the media. Media was autoclaved at 121 °C 1.5 kpa

for 20 minutes. Media were then dispensed into sterile plastic vials containing 20-25 ml of aliquots, inside the laminar air flow cabinet. For culturing of *Pereskia grandifolia* the axillary bud explants were sliced and cultured on MS medium consisted of 0.1-10 mg/l BAP and 30 mg/l adenine as well as BAP and NAA. Petioles obtained from aseptically grown young plantlet of *Gerbera jasmesonii* were used as source of explants. Leaves and petioles were cultured for shoot induction on MS media containing BAP and NAA at various concentrations. Plant regeneration from leaf disk callus of *Begonia elatior* was achieved on MS medium supplemented with 1.0 mg/l Kn and 0.1 mg/l zeatin [17]. Liquid medium seems to be more effective for shoot regeneration and root induction, which is due to better aeration. Simmonds and Werry used liquid medium for enhancing the micropropagation profile of *Begonia hiemalis* [18]. Liquid media have been used for plant cells, somatic embryos and cell suspension cultures in either agitated flasks or various types of bioreactors [19-24]. Wated et al. compared performance of agar-solidified medium and interfacial membrane drafts floating on liquid medium for shoot multiplication and root induction [25]. The regenerated shoots were rooted on half-strength MS medium supplemented with 0.1 mg/l NAA and 0.2 mg/l Kn. Nearly 300 plantlets of each cultivar were transferred to soil with 95% survival rate [26].

5.1.3. Active chemical roles

During *in vitro* conditions, plantlets are grown under fixed and controlled environment in sterile formulated medium which contained macronutrients, micronutrients, vitamins and plant growth regulators. When explants are first placed onto a nutrient medium, there is often an initial leakage of ions from damaged cells, especially metallic cations (Na^+ , Ca^{2+} , K^+ , Mg^{2+}) for the 1-2 days, so that the concentration in the plant tissues actually decreases [27]. Cells then commence active absorption and the internal concentration slowly rises. Phosphate and nitrogen (particularly ammonium) are absorbed more rapidly than other ions. Both growth and morphogenesis in tissues cultures are markedly influenced by the availability of nitrogen and the form in which it is presented [28]. Chemical and substances are synthesized in particular cells and are transferred to other cells, which in extremely small quantities influence the development process. The plant growth regulators are implicated in many biological processes in ornamental plants, including cell division, root and floral initiation, fruit development, senescence and abiotic stress responses.

The rooting efficiency enhanced by addition of 0.05% Poly vinyl pyrrolidone (PVP) in the culture medium containing 0.5 mg/l IBA [29]. The addition of PVP helps in oxidizing polyphenols leached in the medium, and promotes high rate of organogenesis. Dijkshoorn-Dekker studied the influence of light and temperature on propagation profile of *Ficus benjamina* [30]. Propagation of different *Ficus* species by using shoot tips or axillary bud explants had been reported [29,31-34]. In the most cases, shoots were rooted in hormone free medium. Both orientation of the petiole explants and auxin transport system are crucial factors for the induction of somatic embryogenesis of *Saintpaulia* [35], and TDZ helped in the development of somatic embryos. Winkelmann *et al.* used cell suspension culture of *Cyclamen* for rapid development of somatic embryos [36], and later on followed by Hohe et

al. [37], who developed a large scale propagation system of *Cyclamen* from embryogenic cell suspension cultures. Kumari *et al.* developed an efficient protocol for micropropagation of *Chrysanthemum* on MS medium supplemented with 1.0-2.5 μ M TDZ [38]. Castillo and Smith induced direct somatic embryogenesis from petiole and leaf blade explants of *Begonia gracilis* on MS medium supplemented with 0.5 mg/l kinetin and 2% (v/v) coconut water [5]. Kim *et al.* established a large-scale propagation of *chrysanthemum* through bioreactor system, and obtained 5000 plantlets after 12 weeks of culture in 10 \pm l column type bioreactor [39].

6. Results

6.1. Micropropagation

In vitro propagation through meristem culture is the best possible means of virus elimination and produces a large numbers of plants in a short span of time. It is a powerful tool for large-scale propagation of ornamental plants. The term 'meristem culture' specially means that a meristem with no leaf primordial or at most 1-2 leaf primordial which are excised and cultured. The pathway of regeneration undergoes several steps. Starting with an isolated explant, with de-differentiation followed by re-differentiation and organization into meristematic centers. More than 600 million micropropagated plants are produced every year in the world [40]. Micropropagation is one of the few areas of plant tissue culture in which the techniques have been applied commercially. To circumvent these impediments, clonal or vegetative propagation has been deployed for recovering dominant, additive and epistatic genetic effects to select superior genotypes. Plant tissue culture methods offer an important option for effective multiplication and improvement of ornamental plants within a limited time frame.

6.1.1. *Gerbera jamesonii*

Gerbera jamesonii is an ornamental flowering perennial belonging to the Asteraceae family. This plant is very well known to be planted as cut flowers, bedding plant and also pot crops. The *in vitro* shoots were successfully obtained in petiole explants of *Gerbera jamesonii* [10]. The adventitious shoots were observed on MS medium supplemented with BA (1.0 mg/l) and NAA (0.5 mg/l). The developed shoots were subcultured every two week interval for shoot elongation. The elongated shoots were transferred for rooting on MS media with IAA (2.0 mg/l). The developed plantlets were maintained in the following acclimatization conditions : the garden soil (black soil: red soil, 2:1) which gave best result with 86.0 \pm 0.9% survival rates, followed by vermiculite with 73 \pm 1.3% survival rates. In this study, plantlets established from *in vitro* regeneration of *Gerbera jamesonii* were morphologically identical to the mother plant and developed normally and produced flowers after 6 months being transplanted to the greenhouse [41,42].

6.1.1.1. Multiple shoots induction

Leaf and petiole explants were cultured on MS medium for shoot induction containing BAP (0.5-2.0 mg/l) and NAA (0.5-1.0 mg/l). Normal adventitious shoots of *Gerbera jamesonii* were

successively obtained from petiole explants cultured on MS medium supplemented with BAP (2.0 mg/l) and NAA (0.5 mg/l) with 94.3% regeneration rate and 9.3 shoots per explant, followed by BAP (1.5 mg/l) and NAA (1.0 mg/l) with 83.1% regeneration rates and 8.3 shoots per explant. Pierik et al. stated that the addition of strong auxin such as NAA in combination with BAP promoted shoots induction in plant tissue culture [2]. In this study, higher concentration of auxin, NAA (2.0 mg/l) in combination with lower concentration of BAP (1.0 mg/l) showed the lowest shoots regeneration rates (4.6) with the lowest number of shoots (1.6). Son et al. investigated the micropropagation of different plant varieties using the plant buds as explants [43]. They found that the best hormone combination for the *in vitro* initiation of *Gerbera jamesonii* shoots was 3 mg/l BAP + 0.1 mg/l IAA producing 11.29 number of shoots per explant on MS medium.

6.1.1.2. Rooting and hardening

The induced shoots were best rooted on MS media supplemented with BAP (0.1 mg/l) with frequency of 73.7% and 22.1 roots per explant, while Son et. al.[43] found that MS medium supplemented with 2.0 mg/l NAA was the best medium for *in vitro* rooting of the shoots (94.0%). The developed plantlets were maintained in the following acclimatization conditions: the garden soil (black soil: red soil, 2:1) which gave the best results with 86.0 % survival rates, followed by vermiculite with 73% survival rates. The regenerated plantlets failed to survive, when they were cultured in the autoclaved garden soil (black soil: red soil, 2:1). The plantlets established from *in vitro* regeneration of *Gerbera jamesonii* were morphologically identical to the mother plant and developed normally and also produced flowers after 6 months being transplanted to the greenhouse.

6.1.2. *Pereskia grandifolia* Haworth var. *grandifolia*

A protocol for an *in vitro* propagation was developed for the ornamental plant *Pereskia grandifolia* Haworth var. *grandifolia* from axillary bud explants. Optimum multiplication of shoots was achieved on MS [16] medium supplemented with 3.0 mg/l BAP and 30.0 mg/l adenine. Plants were maintained *in vitro* on MS medium while callus were induced on MS basal medium supplemented with the combination of 5.0 mg/l BAP and 5.0 mg/l NAA. The somatic embryogenic callus of the plant species was induced by Chuah and Chan on B5 medium supplemented with 6.0 mg/l 2, 4-D [44,45].

6.2. Organogenesis

Regeneration in plant tissue culture will be successful by maintaining various factors involved, including media factors and environmental factors. The media factors include media constituents, macronutrients, micronutrients, vitamins, amino acids, carbon source, complex nutritive mixtures, gelling agents, activated charcoal, plant growth regulators and pH of the medium. Environmental factors on the other hand are the culture conditions under which explants are maintained. The environmental factors involved include the temperature and illumination of the culture room, agitation process and incubation period of the cultures [4]. For the initiation of callus culture, the following factors are important:-

the origin of explants used for the establishment of callus culture, the cellular/tissue differentiation status, external plant growth regulators, culture media and culture conditions [46]. Cellular competence to plant hormones is understood as the status in which a cell must possess the ability to perceive a transducer and respond to a signal [47]. Organogenesis refers to the formation of shoots/roots. The callus may remain in a differentiated condition regardless of the hormones and nutrients to which it is exposed the secondary metabolites and these metabolites have biological activity [48-50]. Organ formation generally follows cessation of unlimited proliferation of callus. Individual cells or group of cells of smaller dimensions may form small nets of cells scattered throughout the callus tissue, the so-called meristemoids. These meristemoids become transformed into cyclic nodules from which shoot bud or root primordia may grow as shoots/roots. Shoot bud formation may decrease with age and subculture duration of the callus tissue but the capacity of rooting may persist for longer period. In some calli, rooting occurs more often than in other forms of organogenesis. During organogenesis, if the roots are first formed, then it is very difficult to induce adventitious shoot bud formation from the same callus tissue. If the shoots are first formed, it may form roots later on or may remain in rootless condition unless and until the shoots are transformed to another medium or hormone less medium or conditions that induce root formation. In certain cases root and shoot formation may occur simultaneously, but organ connection (vascular connection) between root and shoot primordial is essential for the regeneration of complete plantlet from the culture. Shoot formation followed by rooting is the general feature of organogenesis. The color of callus tissue may remain unchanged during rhizogenesis or may develop yellow pigmentation. During shoot bud formation, the callus tissue generally develops green or pale green pigmentation. Organogenesis is a process by which a cell or group of cells differentiate to form organs. It is the reflection of the intrinsic genetic constitution of a taxon [51]. Since recovery of plants is the usual objective, regeneration of shoots is of greater interest. Organogenesis is commonly induced by manipulation of exogenous phytohormone levels and occurs either directly from explant tissue or through callus.

6.2.1. *Saintpaulia ionantha*

Saintpaulia ionantha known worldwide as African violet belongs to the family Gesneriaceae. African violet is one of the most popular ornamental plants. The complete plant regeneration was obtained from leaf, petiole, peduncle and floral parts of the plant. For shoot regeneration MS medium supplemented with IAA (1.0 mg/l) with Zeatin (2.0 mg/l). The developed plantlets were transferred for acclimatization, whereby *in vitro* plantlets were transferred into planting pots containing mixed soil, which is a mixture of compost, sand and black soil with the ratio of 1:1:2. The morphological characters were compared to the mother plants [52].

6.2.2. Somatic embryogenesis

The commercial exploitation of the ornamental plants for the production and conventional propagation is hampered due to their poor seed viability, low rate of germination and poor rooting ability of the vegetative cuttings. Somatic embryogenesis is an alternative method.

However, there is lack of information for the embryo induction process [53]. The aim of work was to study the germination capability and development of somatic embryos (SEs) from ornamental plants. A sustainable plant regeneration system *in vitro* through somatic embryos from mature sexual embryos has been reported in *Clitoria ternatea*. Somatic embryos developed through callus from seedling roots on hormone-free MS medium (MS₁). Addition of growth hormones, Kn 0.5 mg/l (MS₂) or Kn +IAA 0.5 mg/l of each (MS₃) induced direct somatic embryos, in high frequency, on split root and hypocotyl systems. The embryogenic potential varied with the organ, roots or hypocotyls, and also with the medium. The morphogenetic capacity of the somatic embryos is retained for more than 2 years by sub-culturing at intervals of 4 weeks on MS₃ in complete darkness. Somatic embryos, under the appropriate subculture conditions (16 h light/8 h dark photoperiod at 24± 1 °C on media MS₃, MS₄ and MS₅), resulted in recurrent-somatic embryogenesis and was profuse at the shoot and root apices of the somatic embryos. Mature somatic embryos were transplanted to MS₁ to stimulate germination and plantlet regeneration. Plantlets, developed from primary and secondary embryos on MS₁ were successfully hardened and grown in natural outdoor conditions. The morphology and histology of the somatic embryo and plantlet and the culture conditions for continuous production of plantlets through direct somatic embryogenesis are discussed. In our laboratory, plant regeneration of *Clitoria ternatea* was reported from leaf explants cultured on DKW (Driver and Kuniyuki) medium supplemented with various concentrations of NAA and BAP [54].

Embryogenic callus was induced from leaf explants of *Gerbera jamesonii* Bolus ex. Hook f. in cell suspension cultures. A cream friable embryogenic callus was formed within two weeks when leaf explants were cultured on MS medium containing 2,4-D. hormone (1.0 to 2.0 mg/l). A hundred percent (100%) induction frequency was obtained in 2,4-D concentration range of 1.7-2.0 mg/l. While maximum percentage response for somatic embryos induction (64.56%) from callus clumps was obtained on MS medium fortified with BAP (0.5 mg/l) and NAA (1.0 mg/l) by Ranjan and Gaurav [27]. For proliferation, embryogenic callus was transferred to MS liquid medium containing the same hormone; 2,4-D with a small amount of NAA and subcultured at 2 weeks interval. Induction of somatic embryos different stages (globular, heart and torpedo) were observed after 2 weeks of culture. Somatic embryos were developed in MS suspension medium containing 1.0 to 2.0 mg/l 2,4-D with 0.1 or 1.0 mg/l NAA and the globular embryos were further differentiated into the cotyledonary phase embryos. The addition of 5.0 mg/l amino acids (L-glutamine or L-proline) to the culture media, in the range of the tested medium condition, yielded higher enhancement of the embryo growth and development. Transferring of individual embryos onto a fresh basal MS medium without plant growth regulators enabled the achievement of complete maturation. Relatively, only a small number of the induced embryos developed shoots and roots when they were transferred to MS medium supplemented with 2.0 mg/l BAP and 0.5 mg/l NAA in addition of 3% (w/v) sucrose and 0.8% (w/v) agar. Nearly, 11% of somatic embryos were able to be converted to fertile plants. This similar result was supported by other authors [3,55].

6.2.3. *Dianthus caryophyllus*

The *in vitro* regeneration of the carnation plant species was established from aseptic shoots produced from surface sterilized seeds grown in basal MS medium. High percentage of grown seeds was obtained (50-90%) after 3 weeks of culturing the seeds. All the plantlets were rooted efficiently on the multiplication MS medium without hormone supplementation. This may be due to the presence of endogenous auxins which causes rooting as it was stated by Mosleh et al. [56]. After 4 weeks, full grown plantlets were transferred to sterile soil at ratio of 1:1:1 for garden soil, sand and loam for acclimatization process. They were maintained in the culture room at $25\pm 1^{\circ}\text{C}$ with 16 hours light and 8 hours dark planted in pots and covered with polystyrene plastic bags. The growth was encouraging and after 4 weeks in the culture room, well grown and healthy plantlets were transferred to the greenhouse. Survival rate of the acclimatized plantlets was 78% and it showed that the most of the plantlets successfully survived after transferred to the greenhouse. Best response for hardening of the plant species was also obtained by Aamir et al. [57] (95%) in mixture containing sand, peat and soil (1:1:1) under natural light conditions [58].

6.2.4. *Saintpaulia ionantha*

The complete plant regeneration was obtained from leaf, petiole, peduncle and floral parts of the plant. For shoot regeneration, MS medium supplemented with IAA (1.0 mg/l) and Zeatin (2.0 mg/l) induced the highest number of shoots (15.0) in 8 weeks. While shoots regeneration and multiplication obtained from the same plant by Azura et al. [59] with maximum induction rate on MS medium supplemented with 3.0 mg/l BAP and 1.0 mg/l of NAA. It was reported by Hasbullah et al. [10] that a good combination of cytokinins and auxin in the plant culture medium enhanced good shoot formation and plantlet regeneration. Most of the developed plantlets were hardened (84%) and acclimatized in the greenhouse by transferring the plantlets into planting pots containing mixed soil, which is a mixture of compost, sand and black soil with the ratio of 1:1:2. Meanwhile, Khan et al. found that among the different potting mixes used for the acclimatization of rooted plantlets, 100% sand was found to be the best [59].

However, the obtained new plants failed to flower even after twelve months from planting. The morphological characters of these plantlets were compared to those of the mother plants. They were found to be different in some of their morphological characters such as plant height, leaf size and leaf texture and showed similarity in leaf arrangement and leaf margin and they showed unstable morphological characters. But variations in flowering period, number of flowers per plant and flower morphology were observed by Jain [17] in the plants directly regenerated from leaf disk explants. So, he concluded that the cytokinins, benzylaminopurine and zeatin tested in the culture medium did not affect the basic plant characteristics including flower colour which remained stable in both species. An attempt to induce *in vitro* flowering from african violet was also reported by Daud and Taha [9]. They found that the floral buds were formed *in vitro* with sepal (calyx) and petal (corolla) but did not show any formation of reproductive organs (stamens or pistils).

6.3. Acclimatization

Acclimatization process were carried out while the plants still under *in vitro* condition. A few days before the process was to be carried out, the cover of test tube was removed. With the relative humidity at 50-70% in the culture room, this will increase the epicuticular wax development on the upper leaf surfaces of the plantlets and their survival rate rose from 70 to 90% [60]. The plantlets were exposed to the normal environment in stages as they will wilt due to rapid changes of relative humidity and light intensity. *In vitro* plantlets that reached 3-5 cm height were taken out from culture tubes and the excess media were rinsed to avoid contamination. They were then put into plastic pots and planted out in soil at a ratio of 1:1:1 for garden soil, sand and loam. There were 3 types of treatments which were carried out:

1. Plantlets were planted in a pot and placed a beaker to cover it up.
2. Plantlets were planted in a pot and put in Mistifier device.
3. Plantlets were planted in a pot and covered with transparent plastic lid.

For acclimatization purpose of (*Saintpaulia ionantha*), various substrates were used, such as autoclaved mixed soil (compost, sand, and black soil in the ratio 1:1:2) and non-autoclaved mixed soil. The regenerated plants must reach 4-5 cm before transferring them into pots of mixed soil. After transplanting, the plantlets were watered regularly to prevent from drying. For the first 3 weeks the regenerated plants were maintained in the culture room at 25 ± 2 °C. Gamma irradiations of 10-60 gray were also tested on the regenerated plants to induce flowering and also to observe the effect of radiation on the plantlets. Successful micropropagation of plants which can survive under the natural environmental conditions depends on acclimatization process. Most species grown *in vitro* required an acclimatization process in order to ensure that sufficient number of plants can survive and grow vigorously after being transferred to *ex vivo* soil. The excess media was cleaned from the roots and the plants were transplanted in an adequate substrate such as peat or soil. Plantlets were maintained in a confined environment temporarily before they can be adapted progressively in typical environment within drier air, high light intensity and temperature variations.

6.4. Antimicrobial studies

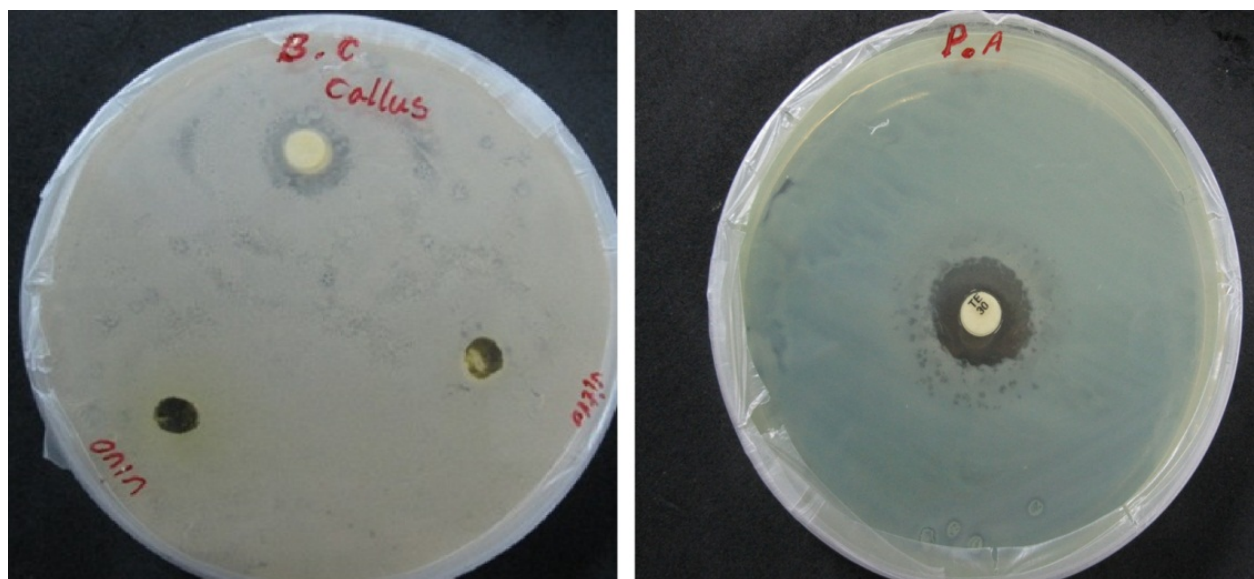
To determine the antimicrobial activity, *Pereskia grandifolia* fresh leaves (300 g) were dried in oven (30-35 °C) for about 5-7 days. Dried leaves were crushed and ground using mortar and pestle in the laboratory. The final weights of the dried powdered materials were 35 g. The leaf powder was extracted with methanol as a solvent. Extracts were filtered and concentrated to dryness using a rotary evaporator. Extract was then ready for the antimicrobials tests. The same procedure was done with aseptic callus to obtain the extract of callus. The antimicrobial screening battery consisted of: gram positive bacterium, *Bacillus subtilis*, *Staphylococcus aureus*; gram negative bacteria, *Escherichia coli*, *Proteus mirabilis*, *Proteus vulgaris*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*; and Fungi, *Candida albicans*, *Microsporium cants*, *Trichophyton rubrum*, *Trichophyton mentagrophyte* and *Aspergillus niger*. The antimicrobials tests were done using standard microbial test culture.

6.4.1. Media for microbial cultivation and maintenance

Meuller Hinton agar, Meuller Hinton Broth, Sabouraud Dextrose Agar (Difco) and Sabouraud Dextrose Broth (Difco) culture media were used. Each medium was prepared to manufacturers' specification and adjusted to the appropriate pH before sterilized by autoclaving at 121 °C for 15 minutes. About 20 ml sterile agar media were poured into petri dishes and let to solidify at a slanted position in Universal bottles. Broth or liquid media were distributed into final containers before autoclaving. Antioxidant and antibacterial activities of ethanolic extracts of *Asparagus officinalis* cv. Mary Washington grown *in vivo* and *in vitro* were compared in our laboratory [61]. Although no antibacterial activity was detected from both *in vivo* and *in vitro* grown plant extracts in the disc diffusion antimicrobial assay, ethanolic extract of *A. officinalis* offered antibacterial activity against *Bacillus cereus* (Table 2; Figure 1).

Plant name	Inhibition Zone of Tested Bactria (mm)			
	E.c.	P.a.	S.a.	B.c.
<i>Zizyphus jujube</i>	-	0.67±0.29	0.5±0.0	-
<i>Thymus vulgaris</i> L.	0.93±0.12	0.5±0.0	2.33±0.58	1.17±0.29
<i>Carum carvi</i> L.	-	0.5±0.0	0.83±0.29	-
<i>Teucrium polium</i> L.	-	0.5±0.0	1.0±0.0	-
<i>Althaea officinalis</i> L.	-	0.5±0.0	1.5±0.5	-
<i>Borage officinalis</i> L	-	-	-	-
Tetracycline (30µg)	13±0.1	2.97±0.06	12.97±0.06	8.07±0.12

Table 1. Inhibition effect of 100 mg/ml of ethanolic extracts of some ornamental plants against the growth of four pathogenic bacteria.



B.c: *Bacillus cereus*; P.a: *Pseudomonas aeruginosa*

Figure 1. Comparison of antibacterial activity of *in vitro*, *in vivo* and callus extracts of *Asparagus officinalis* against *Bacillus cerus* and *Pseudomonas aeruginosa*, using agar diffusion method

Bacteria	Inhibition Zone (mm)			
	<i>In Vivo</i> Plant	<i>In Vitro</i> Plant	Callus	Tetracycline (30 µg)
<i>Escherichia coli</i>	–b	–b	–b	42 ± 3.00 ^a
<i>Staphylococcus aureus</i>	–b	–b	–b	20 ± 2.64 ^a
<i>Pseudomonas aeruginosa</i>	–b	–b	–b	11 ± 2.00 ^a
<i>Bacillus cereus</i>	–c	–c	12 ± 1.00 ^b	40 ± 3.60 ^a

–: No inhibition; The data were analyzed by one-way ANOVA and the inhibition means of samples were compared using Duncan's Multiple Comparison Test (DMCT). Mean of different samples labeled with different letters are significantly different in each row ($p < 0.05$).

Table 2. Inhibition effect of 100 mg/ml of *Asparagus officinalis* ethanolic extracts (*in vivo* plant, *in vitro* plant and callus) against the growth of four pathogenic bacteria

6.4.2. Inoculums for antimicrobial testing

Cultures of fungi and bacteria grown in Mueller Hinton Broth and Sabouraud Dextrose Broth for 18 hours, respectively, were standardized to an optical density of 1 at 600 nm ($OD_{600} = 1$) using NOVASPEC II Visible Spectrophotometer. The density was adjusted by adding sterile broth to the cultures. The concentrations of the resultant suspensions of fungi, bacteria and yeasts were approximately 10^8 cells/ml and 10^7 cells/ml, respectively [62]. The fungi and bacteria suspensions were prepared immediately before carrying out the antimicrobial assay. Conidial suspensions of the test fungi were prepared by pouring 20 ml broth containing 1 drop Tween 80 (Sigma P-8074), into 3-days-old cultures of *Aspergillus* species or 2-week-old culture of *Trichophyton mentagrophytes*. The culture of *Aspergillus* species and *Trichophyton mentagrophytes* were grown on Sabouraud Dextrose Agar slants at 37 °C and 27 °C, respectively. After homogenizing with glass beads, the concentrations of the resultant conidial suspensions of *Aspergillus* species and *Trichophyton mentagrophytes* were adjusted to 10^6 conidia/ml and 10^8 conidia/ml, respectively by using haemocytometer.

6.4.3. Semi-quantitative antimicrobial activity test

According to paper-disk Diffusion assay method, the suspension culture of fungi and bacteria were diluted to the final concentrations of approximately 10^6 cells/ml and 10^5 cells/ml, respectively. The bacterial suspensions were evenly spread on the surface of 4 mm thickness of Mueller Hinton Agar (MHA) plate and the fungi suspensions on Dextrose Sabouraud Agar (DSA) plates. Sterile cotton swabs were used to produce uniform growth of organism. Methanol (MeOH) extracts of the leaves (*in vivo*) and callus (*in vitro*) of *Pereskia grandifolia* were used as test extracts. These extracts were dissolved in methanol and applied to filter disks (Whatman No 1, 6 mm in diameter), at the concentrations of 100, 200, 400 and 800 mg/ml for fungi, yeasts and antibacterial screening. After evaporation of the solvent, the disks were placed in a good contact on the seeded agar plates. Chloramphenicol and 5-fluorocytosine (SIGMA F-7129) at the concentrations of the 1.0 mg/ml were used as positive controls for antifungal and antibacterial, respectively. Saturated filter paper disk of

methanol (MeOH) as blank disks were used as negative controls. Incubation of bacteria and fungi were done at 37°C for 24 hrs. Each extract and control was employed in triplicate for each organism. Diameters of clear zones produced around the disks (if present) were measured after the incubation time. This study showed that the antioxidant and antimicrobial activities showed that these bioactivities differ between *in vitro* and *in vivo* grown plants. Total antioxidant capacity of *in vivo* grown plant was higher than *in vitro* grown plant, while the only antimicrobial activity was obtained from *in vitro* callus tissue against ornamental plants. Some phytochemical studies are required to investigate the production of antioxidant and antimicrobial compounds in differentiated and undifferentiated callus cells of ornamental plants.

6.4.4. Agar diffusion assay

Arnone et al. and Drouhet et al. reported the conidial suspensions of fungi test were diluted 10 times with molten Sabouraud Dextrose Agar at 40 °C and 20 ml was poured into each petri dish [63, 64]. Filter paper disks (Whatman No. 1, 6 mm in diameter) were impregnated with the test extract solutions in methanol (MeOH) at the concentration of 100, 200, 400 and 800 mg/ml. The disks were transferred into the surface of solidified agar after evaporation of solvent. 5-Fluorocytosine at the concentration of 1.0 mg/ml, saturated filter paper disks of methanol as blank disks were applied as controls. Three series of determination were run for each extract and species of fungi. Zones of growth inhibition were measured after 3 days incubation at 37°C for *Aspergillus niger*, whereas a week at room temperature (27 °C) for *Trichophyton mentagrophyte* and *Trichophyton rubrum*. The results of well diffusion antimicrobial activity carried out and showed that the ethanolic extract of *A. graveolens* callus inhibited the growth of *Bacillus subtilis* more than ethanolic extract of the plant roots. Antimicrobial activity of the callus extract might be either related to the production of a compound in only undifferentiated callus cells or may be produced in higher amounts in these cells when compared to differentiated cells. Several quantitative estimations and studies showed that the production of biocompounds can vary between differentiated and undifferentiated plant cells [50,65].

6.5. Future research

- The development of culture methods, particularly those for highly seeping plants, is expected to have a significant environmental benefit by controlling to retention of biodiversity.
- The ornamental and medicinal plants demand for raw materials raises questions surrounding the sustainability of the new industry.
- Development of *in vitro* culture technology is of fundamental importance if ornamental biotechnology is to play a central role in the growth of global ornamental plant production industry in future.
- Ornamental plant tissue culture techniques are expected to be developed enough in the near future when combined with molecular genetics. This may give support to be same

biotechnological applications as in ornamental plant in genomic age, a field in which ornamental plant is also far behind other higher plants.

- Thus intensive work on new strain selection and improvement of an efficient mass culture system is clearly needed. For the exploitation of ornamental plants are developed at the cellular level, plant tissue culture constitutes a basic powerful tool.
- This efficient somatic embryogenesis protocols could be useful for conservation and agronomy and in the improvement of ornamental plants using gene transfer biotechnologies.

7. Conclusion

Successful *in vitro* propagation, organogenesis and somatic embryogenesis with acclimatization of ornamental plants is now being used for commercialization. Many commercial laboratories and national research institutes worldwide use *in vitro* culture system for rapid plant multiplication, germplasm conservation, elimination of pathogens, genetic manipulations and secondary metabolite production. This somatic embryos protocol could be useful for conservation and agronomy and in the improvement of ornamental plants using gene transfer biotechnologies. The results of present investigation clearly indicate that antimicrobial activity vary with the plant species of the ornamental plants and plant material used. Thus, the study ascertains the value of ornamental plants used in Ayurveda, which could be of considerable interest to the development of new drugs.

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